Mapping the human α globin gene complex to 16p13.2 $\rightarrow$ pter

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SUMMARY The regional localisation of the α globin (*HBA*) complex on the short arm of chromosome 16 was investigated by in situ hybridisation, Southern blot analysis, and linkage analysis using the fragile site at 16p12.3 (*FRA16A*) and translocation breakpoints within band 16p13.1. *HBA* was found to be distal to the two translocation breakpoints within band 16p13.1 and not closely linked to *FRA16A*. In situ hybridisation to the distal portion of the fragment on metaphase chromosomes expressing the fragile site and the observation of considerable recombination between *FRA16A* and *HBA* supports a localisation for *HBA* closer to the telomere than to *FRA16A*, at 16p13.2 $\rightarrow$ pter.

The human α globin gene complex, which includes the α and ζ globin genes and their pseudogenes, was assigned to chromosome 16 using somatic cell hybrids¹ and localised to the distal half of the short arm (16p12 $\rightarrow$ pter) by in situ hybridisation.² Components of the α globin (*HBA*), phosphoglycolate phosphatase (*PGP*), adult polycystic kidney disease (*PKDI*) linkage group³ have been localised further. *PGP* was placed at 16p13 using somatic cell hybrids⁴ and *HBA* was placed at two conflicting locations, 16p13.1⁵ and 16p13.3,⁶ using chromosomal rearrangements.

This study uses the rare folate sensitive fragile site *FRA16A* (fra(16)(p12)) at 16p12.3 and the breakpoints of two constitutional chromosomal rearrangements to support the more distal localisation of the *HBA* complex on the short arm at 16p13.3.

Materials and methods

IN SITU HYBRIDISATION

Lymphoblastoid cell lines were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). GM2324 has a t(16;22) with breakpoints at 16p13 and 22q12 which were redefined as 16p13.11 and 22q11.21 by Nicholls *et al.*⁵ GM6227 has a karyotype reported as 46,XX,t(1;16)(q44;p13.11). Cells from a subject 46,XY, fra(16)(p12) were obtained from a family previously studied.⁷

Chromosome preparations expressing the fragile site *FRA16A* in 60% of metaphases were made from blood lymphocytes.⁸ Chromosomes from the translocation cell lines were G banded with trypsin for identification, photographed, and destained before in situ hybridisation.

JW101 consists of α globin cDNA in the plasmid pMB9⁹ and hybridises with the $\alpha 2$ and $\alpha 1$ sequences. pHP ζ consists of the plasmid pBR322 with a 400 bp insert consisting of part of intron 1 and all of exon 2 of the $\psi\zeta$ globin gene (T Maniatis, 1983, personal communication). p $\alpha 3'$ HVR.64³ consists of a 4.0 kb insert with a tandem repeat array, from the hypervariable region 8 kb downstream to the coding sequences of the α globin genes, in the plasmid pSP64.

In situ hybridisation was carried out as described in Simmers *et al.*,¹⁰ except that chromosomes were denatured in 70% formamide, 2 $\times$ SSC at pH 7.0 and 70°C, and hybridisation was at 37°C. Chromosomes probed with JW101 and pHP ζ were developed 50 to 83 days after dipping in Kodak NTB-2 emulsion diluted 2:1 with water, while those probed with p $\alpha 3'$ HVR.64 were developed after 14 days' exposure.

SOUTHERN BLOT ANALYSIS

Southern blot analysis, using JW101 and p $\alpha 3'$ HVR.64 to localise these probes and determine the recombination frequency between 3'HVR and *FRA16A*, was carried out using a modification of the method of Wahl *et al.*,¹¹ as described by Mulley *et*

FIG 1 Family segregating for *FRA16A* with 3' HVR genotypes. Genotype nomenclature is explained in the text.

al.¹² Autoradiographs were exposed for five to 18 hours. Hybrid line CY18 contains a complete chromosome 16 as the only human chromosome, hybrid line CY3 (derived from GM3884) contains der(16)t(X;16)(q26;q24), effectively chromosome 16 deleted for 16q24, as the only human chromosome, and hybrid line CY13 (derived from GM6227) contains der(16)t(1;16)(q44;p13·1), effectively chromosome 16 deleted for p13·1→pter, as the only human chromosome.¹³

LINKAGE ANALYSIS

Part of the family segregating for the rare folate sensitive fragile site *FRA16A* (fig 1) has been previously described.⁷ This fragile site is situated in the middle of band 16p12·3. The 3' HVR probe detects numerous alleles within the 1 to 8 kb range when DNA is restricted with *Pvu*II.³ Alleles for the family studied were named alphabetically A, B, C, D, and E in order of decreasing molecular weight. Allele class D, associated with DNA fragments of approximately 3 kb, consisted of both suspected and clearly visible doublets. Separation was initially inadequate for definite classification of all subjects. Many subjects in the pedigree critical for linkage analysis were of this ambiguous phenotype. Southern analysis was repeated for those with D alleles by extended electrophoresis in 1% agarose gels, running subjects with similar molecular weight bands side by side. The classification of D subtypes was confirmed using *Rsa*I which generates smaller fragments than *Pvu*II, enhancing separation. This allowed the subdivision of D into D1, D2, D3, and D4 in order of decreasing molecular weight, with D2 and D3 the most difficult to separate. Genotypes comprising eight alleles, A, B, C, D1, D2, D3, D4,

and E are shown in fig 1. Data were analysed using the computer programme LIPED.¹⁴

Results

IN SITU HYBRIDISATION

In situ hybridisation showed the *HBA* complex to

FIG 2 An idiogram of the normal and derived chromosomes 1 and 16 from GM6227, showing the position of silver grains from 62 relocated metaphases after in situ hybridisation with 0.4 µg/ml JW101.

distal to the two breakpoints in band 16p13 in the cell lines GM2324 and GM6227 and to *FRA16A*. There were excesses of silver grains on the derived chromosomes which include the part of chromosome 16 distal to the breakpoints in the two cell lines (figs 2 and 3).

FIG 3 An idiogram of the normal and derived chromosomes 16 and 22 from GM2324, showing the position of silver grains from 102 relocated metaphases after *in situ* hybridisation with 0.2 μ g/ml *pa3'*HVR-64.

The silver grains on 16p resulting from hybridisation to chromosomes expressing *FRA16A* were scored either proximal or distal to this fragile site, with the exception of those touching both regions across the gap ('central' grains). There was an excess of silver grains distal to the fragile site for both the α and ζ globin probes (fig 4a, b).

In addition, the position of all silver grains located on the acentric fragment distal to *FRA16A* (16p12.3 $\rightarrow$ pter), including 'central' grains, were analysed further in cells where the orientation of this fragment was clear. Grains were determined to be either closer to the proximal or distal ends of the fragment, but in some cases they were near to the mid-point and such a distinction could not be made, so were excluded from the analysis (fig 5). There were 108 silver grains over the distal half of the acentric fragment, 69 grains on the proximal part of this fragment, and 32 grains for which such a distinction could not be made (fig 4c). The mid-point of the acentric fragment is within band 16p13.2. The significant excess of silver grains on the distal half of the acentric fragment ($p < 0.005$, fig

FIG 4 Idiograms showing the localisation of silver grains scored (a) with respect to 73 *FRA16A* after hybridisation with 0.8 μ g/ml of the α globin probe; (b) with respect to 103 *FRA16A* after hybridisation with 0.8 μ g/ml of the ζ globin probe; (c) on the distal acentric fragment (*fra*(16) (p12.3 $\rightarrow$ 16pter)) of 171 *FRA16A* after hybridisation with the α and ζ globin probes. In each case the assumption that the two classes of silver grains were equal was tested. (a) $\chi^2 = 8.00$, $p < 0.005$, (b) $\chi^2 = 9.94$, $p < 0.005$, (c) $\chi^2 = 8.59$, $p < 0.005$.

FIG 5 Examples of chromosomes 16, expressing *FRA16A*, illustrating silver grains (a) distal; (b) near the mid-point and not clearly closer to either end; and (c) proximal on the acentric fragment (*fra(16)(p12-3)→16pter*). The figure is also illustrated as an outline diagram, as photographic reproduction diminishes the clarity of the image. All chromosomes shown have identical magnification.

FIG 6 Localisation of 3' HVR (probed with *pa3' HVR-64*) to 16p13 by hybridisation to CY18 (lane 2), CY3 (lane 5), human genomic DNA (lane 3), but not to CY13 (lane 6) or mouse genomic DNA (lane 1). 16p13 is deleted from CY13 (derived from GM6227) by a breakpoint in 16p13.1.

4c), therefore, indicates that the *HBA* complex is likely to be closer to 16pter than to *FRA16A*, at 16p13-2→pter.

SOUTHERN BLOT ANALYSIS

The α globin and 3'HVR probes hybridised to the hybrid cell line containing a complete human chromosome 16 (CY18) but not to the hybrid cell line from which 16p13 was absent (CY13) (fig 6). This agrees with in situ hybridisation, placing the *HBA* complex distal to *FRA16A* and distal to the breakpoint within band 16p13 in the cell line GM6227.

LINKAGE ANALYSIS

Recombination associated with the physical separation between 3'HVR and *FRA16A* provides an estimate of the map distance between them. 3'HVR genotypes from a large kindred segregating with *FRA16A* are shown in fig 1.

Extensive recombination was observed between the *HBA* complex and *FRA16A* ($\theta=0.24$, $\hat{Z}=0.38$) (table) using only that portion of the pedigree to which data were complete. Complete data were available for the portion comprising III.1, III.2, IV.1 to IV.6, and V.1 to V.5 and it alone has nine informative meioses. There are at least two definite recombinants from nine opportunities (V.2 and either IV.5 or IV.1, IV.3, and IV.4). The D1 subtypes in this portion of the pedigree were D2 and D4, both of which could be unambiguously separated. Apparent recombination is not an artefact of non-penetrance for the fragile site because both V.4 and IV.5 (the two likely recombinants) were fragile site positive. The remaining portion of the pedigree was not included in the analysis because of uncertainty of genotypes from missing subjects in the first three generations, but additional recombination events are evident by inspection of the extended pedigree (fig 1).

More linkage data are necessary for a precise estimate of recombination frequency. Unfortunately

TABLE Lod scores from linkage analysis using that portion of the pedigree where data are complete (III.1, III.2, IV.1 to IV.6, V.1 to V.5).

	θ	0.01	0.05	0.1	0.2	0.3	0.4	$\hat{\theta}$	$\hat{Z}$
Part pedigree		-1.62	-0.35	0.09	0.36	0.35	0.22	0.24	0.38

large families with *FRA16A* are very rare. Existing linkage data between 3'HVR and *FRA16A* are inadequate for supporting a regional localisation, but clearly show that the *HBA* complex is not closely linked to *FRA16A*.

Discussion

The results of in situ hybridisation and Southern analysis place the *HBA* complex distal to the breakpoints defined at 16p13.11 in the cell lines GM6227 and GM2324, in agreement with the results of Nicholls *et al*⁵ using the same cell lines. Nicholls *et al*⁵ and Breuning *et al*⁶ have localised the *HBA* complex to conflicting regions: bands 16p13.1 and 16p13.3, respectively. Both studies used chromosome rearrangements involving the short arm of chromosome 16 with associated high resolution chromosome banding techniques. The cytogenetic analysis of such chromosome rearrangements is often difficult, especially when 16p is involved. *HBA* lies proximal to a breakpoint on 16p defined as being at 16p13.1 in an inv(16)⁵ and distal to a breakpoint defined as being at 16p13.3 in a t(4;16).⁶ Clearly these two localisations of *HBA* are in conflict, probably because at least one of the breakpoint localisations is incorrect.

Nicholls *et al*⁵ recognise a three band uncertainty for breakpoint localisation proposed by Savage,¹⁵ that is, that the designated location of a breakpoint may be incorrect by one band in either direction. If this uncertainty is taken into account at the level of band resolution obtained, then the wider limits of localisation of the *HBA* complex are 16p12.3→p13.2 from the data of Nicholls *et al*⁵ and 16p13.2→pter from the data of Breuning *et al*.⁶ These two regions overlap. However, this does not necessarily indicate that the breakpoints involved in the two rearrangements in question are both within subband 16p13.2. Depending on the clarity and similarity of bands on both chromosome regions involved in any rearrangement, the uncertainty may be wider than three bands.

The *FRA16A* marker is fixed at a clearly defined location, whereas the sites of breakage in constitutional chromosome rearrangements involving 16p may be difficult to pinpoint. The precise location of the fragile site provides a clear advantage in gene localisation. The *HBA* complex is clearly distal to *FRA16A*, in agreement with the conclusions of Nicholls *et al*⁵ and Breuning *et al*.⁶ It was therefore of interest to attempt sub-localisation of the *HBA* complex, scoring silver grains obtained from in situ hybridisation closer to either of the two precise markers, the fragile site or the telomere. The results

indicated that the *HBA* complex is likely to be closer to 16pter, and therefore within the region 16p13.2→pter.

Nicholls *et al*⁵ also describe in situ hybridisation experiments with a biotin labelled probe, where the majority of label is reported to be over band 16p13.1. Resolution using a biotin labelled probe should be higher than that obtained with tritium, assuming that the resolution of the chromosome banding is high enough to allow accurate band designation of label at the reported level. Again, the fragile site offers a clear and precise reference point on 16p for in situ hybridisation.

Penetrance of autosomal folate sensitive fragile sites, collectively analysed, appears to be 100% when transmitted by females and 50% when transmitted by males.¹⁶ Non-penetrant subjects may bias upwards the estimates for recombination frequency between *FRA16A* and 3'HVR, though in fig 1 V.2, III.9, and III.10 have involved definite recombination and III.2 and IV.5 are probable recombinants between 3'HVR and *FRA16A*. All have the fragile site, so apparent recombination cannot be an artefact of incomplete penetrance.

Three of the five offspring of carrier males in the portion of the pedigree analysed had the fragile site. If IV.3 and V.1 were non-penetrant carriers, recombination would be even greater than that already noted. This is the only *FRA16A* family ever subjected to segregation analysis, so in the absence of firm data on possible incomplete penetrance for this fragile site, penetrance figures were not incorporated into the linkage analysis.

There is no firm evidence that folate sensitive fragile sites affect recombination frequency,^{17 18} though one, *FRAXA*, is located in a telomeric chromosomal region associated with high recombination,¹⁹ and another, *FRA16A*, is located on a chromosome arm where chiasmata are normally observed more frequently than expected on the basis of physical length.²⁰ The exact relationship between physical and genetic length is at present unclear for the short arm of chromosome 16, but if the map of Cook *et al*,²⁰ based on observed chiasmata, is correct then a recombination frequency of 24% between 3'HVR and *FRA16A* is consistent with the more distal localisation of the *HBA* complex, that is, to 16p13.2→pter. However, the confidence limits for this estimate are wide and the regional localisation suggested in this study rests on the results of in situ hybridisation.

A precise location for the *HBA* complex is crucial for continued development of new markers linked to *PKDI*. Closely linked markers will be applicable to clinical diagnosis and will eventually provide a means to clone the *PKDI* gene indirectly and

determine the pathogenesis of the disease by reverse genetics.

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